Stratum Corneum Tryptic Enzyme in Normal Epidermis: a Missing Link in the Desquamation Process?[1]

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Stratum corneum chymotryptic enzyme may be important in desquamation. It has also been suggested that other proteases, especially stratum corneum tryptic enzyme, may be involved. Stratum corneum tryptic enzyme has been purified and its cDNA has been cloned. Results from expression analyses indicate that stratum corneum tryptic enzyme is as skin specific as stratum corneum chymotryptic enzyme. In this work we have produced and characterized antibodies specific for stratum corneum tryptic enzyme. We have also by means of biochemical, immunochemical, and immunohistochemical methods performed studies on stratum corneum tryptic enzyme in normal human epidermis. Antibodies against bacterial recombinant stratum corneum tryptic enzyme were produced and purified by affinity chromatography. Two types of antibodies were obtained: one reacting only with pro-stratum corneum tryptic enzyme and one specific for the catalytically active part of stratum corneum tryptic enzyme. Immunohistochemistry with the antibodies reacting with pro-stratum corneum tryptic enzyme showed a staining pattern similar to stratum corneum chymotryptic enzyme-specific antibodies, i.e., the expression was confined to cornifying epithelia with a need of desquamation-like processes. Extracts of tape strips with superficial human stratum corneum were found to contain precursors as well as active forms of stratum corneum tryptic enzyme and stratum corneum chymotryptic enzyme. The enzymes had maximal activity at pH 8, but both had considerable activity also at pH 5.5. The results were compatible for a role of stratum corneum tryptic enzyme in desquamation. Stratum corneum tryptic enzyme may act in concert with stratum corneum chymotryptic enzyme and/or function as a stratum corneum chymotryptic enzyme-activating enzyme. The presence in normal superficial stratum corneum of precursors as well as of active forms of stratum corneum chymotryptic enzyme and stratum corneum tryptic enzyme, and the activity of both enzymes over a broad range of pH-values, suggest some possible ways by which the desquamation may be regulated. ***Key words: cornification/proteases/stratum corneum chymotryptic enzyme/stratum corneum. J Invest Dermatol 114:56–63, 2000***

Desquamation, i.e., shedding of cornified cells from the skin surface, is a well regulated process. The mechanisms involved are still poorly understood. In recent years, however, it has become clear that one of the mechanisms involved in desquamation is proteolysis of intercellular adhesive structures in the stratum corneum (Bisset *et al*, 1987; Egelrud *et al*, 1988; Lundström and Egelrud, 1988, 1989; Egelrud and Lundström, 1989; Chapman and Walsh, 1990). Stratum corneum chymotryptic enzyme (SCCE) may be involved in this process by degradation of intercellular parts of desmosomes (Egelrud and Lundström, 1991; Lundström and Egelrud, 1991). Results from previous studies have shown enzymatic properties (Egelrud and Lundström, 1991; Lundström and Egelrud, 1991; Egelrud, 1993) and a tissue localization (Egelrud, 1992; Sondell *et al*, 1995) compatible with this function.

There is also evidence of involvement of other proteases in desquamation. As SCCE is produced as an inactive precursor with no proteolytic activity, there is a need for an activating enzyme. This activation involves a proteolytic cleavage of the SCCE propeptide by an enzyme with a trypsin-like substrate specificity (Hansson *et al*, 1994).

On zymograms of extracts of stratum corneum, caseinolytic components with an apparent molecular mass about 30 kDa can be detected in addition to SCCE (Lundström and Egelrud, 1991; Egelrud, 1993). This 30 kDa protease can be inhibited by leupeptin but not by chymostatin (Egelrud, 1993), suggesting a trypsin-like primary substrate specificity. Suzuki *et al* (1993, 1994) presented evidence that desquamation may be dependent on both a chymotrypsin-like and a trypsin-like enzyme, and they also suggested that the trypsin-like protease involved could be the 30 kDa component detected on zymograms.

The 30 kDa protease has been purified from human plantar stratum corneum and its cDNA has been cloned. In reduced form the purified protein has an apparent molecular mass of about 33 kDa. Its cDNA is encoding a 293 amino acid residues long protein consisting of a 29 amino acid residues long signal peptide, a 37 amino acid residues long propeptide, and a 227 amino acid

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Abbreviations: SCCE, stratum corneum chymotryptic enzyme; SCTE, stratum corneum tryptic enzyme.

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Table I. Oligonucleotides used for construction of plasmids for expression in *E. coli* of fusion proteins containing SCTE-derived polypeptides[a]

Oligomer	5′–3′ sequence	*scte*-cDNA[b]
LJ1	gac gac gac aag atg **aac aat gat gtt tcc tgt gac**	(–110)–(–90)
LJ3	gac gac gac aag atg **atc atc aat gga tcc gac tgc gat**	1–24
rLJ4	gag gag aag ccc ggt **tca gga gtt ggc ctg gat ggt tt**	684–662

[a]The sequences shown in bold are specific for SCTE, the nonbold sequences are vector derived.
[b]Positions in *scte* cDNA from (Brattsand and Egelrud, 1999).

Table II. Preparations of SCTE-specific antibodies

Antibody	Rabbit	Time of bleeding	Affinity gel ligand
Pe-1	Pe	2 wk after 2nd injection	None (whole serum)
Pe-C1	Pe	2 wk after 2nd injection	Recombinant pro-SCTE
Br-1	Br	2 wk after 4th injection	None (whole serum)
Br-B1	Br	2 wk after 4th injection	Recombinant SCTE
Br-C10	Br	2 wk after 4th injection	Recombinant pro-SCTE (antibodies purified from break-through fraction in purification of Br-B1)

residues long protein corresponding to the catalytically active enzyme. The deduced amino acid sequence is compatible with a cDNA encoding a serine proteinase with a trypsin-like substrate specificity. Results from expression analyses of a number of human tissues indicated that this new enzyme, tentatively called stratum corneum tryptic enzyme (SCTE), may be as skin specific as SCCE (Brattsand and Egelrud, 1999).

In order to characterize SCTE further, we have produced and characterized antibodies to SCTE. The antibodies were used in immunohistochemical and immunochemical analyses, in addition to biochemical analyses, of normal human epidermis and stratum corneum. The results suggest that SCTE may participate in the desquamation process, either by acting together with SCCE in the degradation of intercellular cohesive structures in the stratum corneum, or as an activating enzyme for other proteases such as SCCE.

MATERIALS AND METHODS

Production of fusions proteins and immunization Total RNA was isolated from HaCaT cells (a gift from Dr. N.E. Fusenig, Heidelberg, Germany; Boukamp *et al*, 1988) using TRIzol (Life Technologies, Täby, Sweden) as recommended by the manufacturer. cDNA was synthesized from 1 μg of total RNA using the Ready to Go T-primed First-Strand Kit (Pharmacia Biotech, Sollentuna, Sweden). The cDNA was polymerase chain reaction amplified using the primer pairs shown in **Table I**.

For amplification of cDNA corresponding to active SCTE the primer pair LJ3–rLJ4, and for cDNA corresponding to pro-SCTE, primer pair LJ1–rLJ4 was used. Polymerase chain reaction was performed according to standard protocols with Taq polymerase purchased from Boehringer Mannheim (Mannheim, Germany). The polymerase chain reaction was run for 30 cycles with an annealing temperature of 65°C. The polymerase chain reaction products were separated on NuSieve DNA Agarose gels (FMC Bioproducts, Rockland, ME). Separated DNA fragments were extracted with the JETSORB kit (Saveen Biotech AB, Malmö, Sweden), treated with T4 DNA polymerase from the PET32-LIC Kit (Novagen, Madison, WI) and annealed into the PET30 vector (Novagen). The constructs were then used to transform *Escherichia coli* Nova Blue cells as recommended by the manufacturer (Novagen). Plasmid DNA was prepared using the Qiagen QIAprepSpin Plasmid Kit (Qiagen, Chatsworth, CA). Nucleotide sequences were verified using the ABI Prism DNA sequencing kit and an ABI 377 automated DNA sequencer (Perkin Elmer, Foster City, CA).

For protein production, the plasmid constructs were used to transform competent *E. coli* BL21(DE3) cells (Novagen). The cells, cultured at 37°C, were induced at $A_{600} = 0.5$ with isopropyl β-D-thiogalactoside at a final concentration of 1 mM, and harvested after 2 h. Recombinant proteins, extracted in 6 M urea from insoluble material recovered from cells lysed by sonication in phosphate-buffered saline (PBS), were purified on a His-Bind Ni column (Novagen) with 6 M urea in the buffers. Crude extracts of PBS-insoluble material solubilized in deionized 8 M urea were used for preparation of immunoaffinity columns (see below).

Two rabbits were immunized. Immunization was carried out with the fusion protein containing the pro-SCTE polypeptide. Recombinant protein recovered from the Ni-column was precipitated from elution buffer containing urea by dialysis against water. The precipitate was homogenized in Freund's complete adjuvant and used for immunization. Protein for immunization was also prepared by separating eluted extracts from the Ni column by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE). Gels were stained with Coomassie Blue, gel slices containing recombinant protein were destained in 5% HAc and 5% glycerol, homogenized in adjuvant as above, and used for immunization.

Antibody purification For the preparation of affinity gels crude extracts of PBS-insoluble material from lysed bacteria solubilized in 8 M urea, 0.1 M $NaHCO_3$, containing either a fusion protein consisting of a polypeptide corresponding to SCTE, or a polypeptide corresponding to pro-SCTE, were mixed with Affigel 10 (BioRad, Sundbyberg, Sweden). After coupling the gels were washed with 8 M urea followed by PBS, and packed into columns. For each preparation of immunoaffinity gel, urea-soluble material corresponding to 90 ml of culture suspension in a volume of approximately 5 ml was used for coupling to 5 ml of sedimented gel.

For each run 20 ml of rabbit serum was diluted 10-fold in PBS before being applied on the columns. After washing with PBS bound antibodies were eluted with 0.3 M acetic acid. The eluate was immediately neutralized with 1 M Tris–HCl, pH 8, bovine serum albumin (final concentration 1 mg per ml) was added, and pooled fractions were dialyzed against PBS. Sodium azide (0.1%) was added and the purified antibodies stored at +4°C. The designation and type of purification for the antibody preparations are shown in **Table II**.

SCTE and SCCE-containing preparations for antibody characterization Ten grams of plantar stratum corneum was incubated in 200 ml of 0.1 M Tris–HCl, 5 mM EDTA, pH 8, for 15 h at 37°C (Egelrud, 1993). The suspension formed was further homogenized mechanically and insoluble material collected by centrifugation. The pellet was washed once in 200 ml of 0.1 M Tris–HCl, pH 8, and then extracted with 40 ml of 1 M acetic acid for 1 h at room temperature. After centrifugation the extract was passed through a 0.45 μm Minisart N membrane (Sartorius, Göttingen, Germany). It was then either dialyzed against 0.01 M acetic acid and lyophilized in aliquots, or subjected to reversed phase chromatography as described (Brattsand and Egelrud, 1999). Fractions containing SCTE and SCCE [the two enzymes, which co-elute in the chromatography system used (see Brattsand and Egelrud, 1999), were identified by means of zymography] were lyophilized.

Extracts of tape strips Fifteen centimeter pieces of tape (3M Polyester Tape Trans 12.7 mm, 3M St Paul, MN) were applied on the backs of five volunteers. For the analyses the first strip taken at a given site was used. Single strips were divided into two equal pieces which were placed with nonadhesive sides in contact in sealed plastic bags, approximately 9 × 2 cm, with 1 ml of 1 M acetic acid. The bags were left at room temperature for 15 h. The extract was recovered and the bags washed with 0.5 ml of 0.1 M

acetic acid. Extracts and washings from single tapes were combined and lyophilized. To prepare extracts containing soluble proteins remaining on the tapes after acetic acid extraction 0.5 ml of electrophoresis sample buffer (Laemmli, 1970) with SDS and mercaptoethanol was added to the bags, which were again sealed and incubated for 15 h at 37°C.

Electrophoresis, zymography, and immunoblotting Lyophilized samples were solubilized in electrophoresis sample buffer with SDS but no mercaptoethanol (1/10 of original volume for crude extracts and chromatography fractions, 30 μl for extracts of tape strips). Zymography in casein-containing polyacrylamide gels was carried out as described (Horie *et al*, 1984; Egelrud and Lundström, 1991) with unreduced samples. For electrophoresis and immunoblotting aliquots were supplemented with 1% mercaptoethanol and heated to 90°C for 2 min.

SDS–PAGE (Laemmli, 1970) and immunoblotting (Towbin *et al*, 1979) to polyvinylidene fluoride (PVDF) membranes (Immobilone P, Millipore AB, Sundbyberg, Sweden) were carried out according to routine procedures. Bound antibodies were detected either with alkaline phosphatase-conjugated secondary antibodies and AP Conjugate Substrate Kit (BioRad, Dakopatts, Älsjö, Sweden), or with horseradish peroxidase-conjugated secondary antibodies and the ECL + kit (Amersham Pharmacia Biotech, Solna, Sweden). For extracts of tape strips the same PVDF membrane was used for three consecutive labelings with primary antibodies; in the two first labelings peroxidase-conjugated secondary antibodies were used, followed by a third labeling with alkaline phosphatase-conjugated antibodies. Between labelings the membrane was stripped according to instructions provided by the manufacturer of the ECL + kit.

Antibody adsorption experiments In all experiments the antibody dilutions were chosen so as to give a weak but clearly detectable signal in control experiments with no adsorbent. For adsorption with recombinant pro-SCCE (Hansson *et al*, 1994) serially diluted (final concentrations 0.06–60 μg per ml) pro-SCCE in PBS, 1 mg per ml of bovine serum albumin, was mixed with antibodies diluted in the same buffer. The mixtures were incubated at 4°C for 16 h, centrifuged at 10,000 × *g* for 10 min, and analyzed for immunolabeling capacity on sections of normal interfollicular epidermis by immunofluorescence microscopy.

For adsorption with components from plantar stratum corneum, lyophilized fractions from reversed phase chromatography (see above) containing SCTE and SCCE as revealed by zymography and immunoblotting, were resolubilized in electrophoresis sample buffer and subjected to SDS–PAGE. Separated proteins were electrophoretically transferred to PVDF membranes which were stained with Coomassie Blue or labeled with antibodies as above. A piece of dried Coomassie-stained membrane corresponding to one electrophoresis lane was divided into seven horizontal strips (see **Fig 3*B***). Each strip was cut into two equal pieces. The membrane pieces were wet in methanol for 5 s, blocked for 2 h with 3% dry milk in 0.1 M Tris–HCl, pH 7.5, 0.5 M NaCl, 0.02% Tween-20 (TBS-T), and then incubated with appropriately diluted antibody Pe-C1 or SCCE-specific antibodies. After incubation at 4°C for 24-96 h the strips were removed, and the supernatants were analyzed for remaining immunolabeling capacity with immunofluorescence or immunoperoxidase methods. For repeated adsorption experiments the strips were washed in 1 M acetic acid and rinsed in TBS-T before being re-blocked with milk and used as above.

Immunohistochemistry Four millimetre punch biopsies were taken under local anesthesia from skin of the scalp and the chest of six and nine volunteers, respectively, with healthy skin. For immunoperoxidase staining the biopsies were fixed for at least 15 h in 4% buffered formaldehyde and paraffin-embedded according to routine procedures. Immunoperoxidase staining with affinity purified polyclonal SCTE-specific (Pe-C1) and polyclonal SCCE-specific antibodies was carried out as described (Sondell *et al*, 1996; Ekholm and Egelrud, 1998a, b). For immunofluorescence analyses biopsies were fixed for 2 h in 4% buffered formaldehyde before cryosectioning and staining as previously described (Ekholm and Egelrud, 1998a).

The SCCE-specific polyclonal antibodies were provided by Symbicom AB (Umeå, Sweden). For negative controls IgG prepared from normal rabbit serum (Sondell *et al*, 1996) was used. The detection system (supersensitive StrAviGen multilink horseradish peroxidase and the liquid DAB substrate pack) was purchased from BioGenex (San Ramon, CA).

RESULTS

Characterization of antibodies to SCTE comparison with anti-SCCE **Figure 1** shows immunoblots of a reduced sample prepared from a crude extract of plantar stratum corneum. The

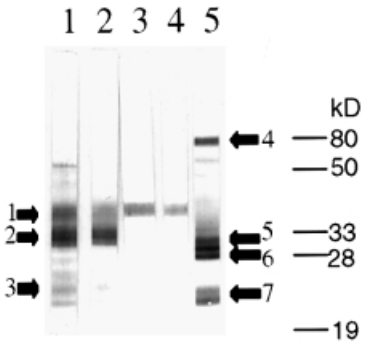

Figure 1. Immunoblot labeling patterns of antibodies to SCTE and SCCE. Crude extract corresponding to 0.25 g of dried plantar stratum corneum was resolubilized in 100 μl of electrophoresis sample buffer and subjected to SDS–PAGE under reducing conditions. Separated proteins were transferred to a 6 × 9 cm PVDF membrane which was then divided into strips used for the labeling experiments. Bound antibodies were detected with alkaline phosphatase-conjugated secondary antibodies. Primary antibodies were: strip no. 1: Br-1, 1/1000; strip no. 2: Br-B1, 1.8 μg per ml; strip no. 3: Br-C10, 0.7 μg per ml; strip no. 4 Pe-C1, 0.6 μg per ml; strip no. 5 anti-SCCE, 0.6 μg per ml. *Arrows* with numbers depict the proposed identity of labeled components: 1, pro-SCTE, molecular mass ≈37 kDa; 2, SCTE, molecular mass ≈33 kDa; 3, proteolytically modified SCTE; 4, complex between SCCE and α1-anti-trypsin, molecular mass ≈80 kDa; 5, glycosylated pro-SCCE, molecular mass ≈30 kDa; 6, unglycosylated SCCE, molecular mass ≈28 kDa; 7, proteolytically modified SCCE. The band between components 5 and 6 in strip no. 5 contains a mixture of unglycosylated pro-SCCE and glycosylated SCCE. Mobilities of reference proteins to the right.

whole rabbit serum Br-1 labeled two groups of components with molecular masses of about 33 kDa and 37 kDa, respectively (*lane 1*). In addition some components with lower molecular mass were labeled. Antibodies prepared from Br-1 by affinity purification on a gel with a fusion protein containing the polypeptide corresponding to active SCTE labeled the same components as the whole serum (antibody Br-B1, *lane 2*).

From serum Br-1 which had previously been passed over a column with the SCTE fusion protein, antibodies labeling only the 37 kDa components could be purified on a gel with a fusion protein containing a polypeptide corresponding to pro-SCTE (antibody Br-C10, *lane 3*). The whole rabbit serum Pe-1 labeled only the 37 kDa-components (not shown), as did antibodies purified from this serum on pro-SCTE fusion protein (antibody Pe-C1, *lane 4*).

For comparison the labeling pattern of the same electrophoresed sample by SCCE-specific antibodies is shown in **Fig 1** (*lane 5*). These antibodies labeled a group of components with molecular masses of about 28–30 kDa, previously identified as glycosylated and unglycosylated pro-SCCE and active SCCE. In addition, a larger component, previously identified as a complex between SCCE and α1-anti-trypsin (T. Egelrud, unpublished observation), and components with molecular masses of about 20 kDa, probably proteolytically modified SCCE, were labeled.

With unreduced samples the labeling of immunoblots with antibodies with suggested specificity for SCTE and pro-SCTE was much weaker, and could be detected only when the antibody concentrations were increased 10-fold. With antibodies Br-B1 and Pe-C1 the detected unreduced components had molecular masses of about 30–34 and 34 kDa, respectively. No components with lower molecular masses were labeled. The labeling with the SCCE antibodies was approximately of the same intensity for reduced and nonreduced samples. The unreduced components detected had molecular masses of about 25-27 kDa (results with unreduced samples not shown).

Analyses of tape strips with superficial stratum corneum Results of analyses of extracts of 15 × 1.3 cm tape strips of superficial normal stratum corneum from five individuals are shown in **Fig 2**. Analyses were carried out by immunoblotting

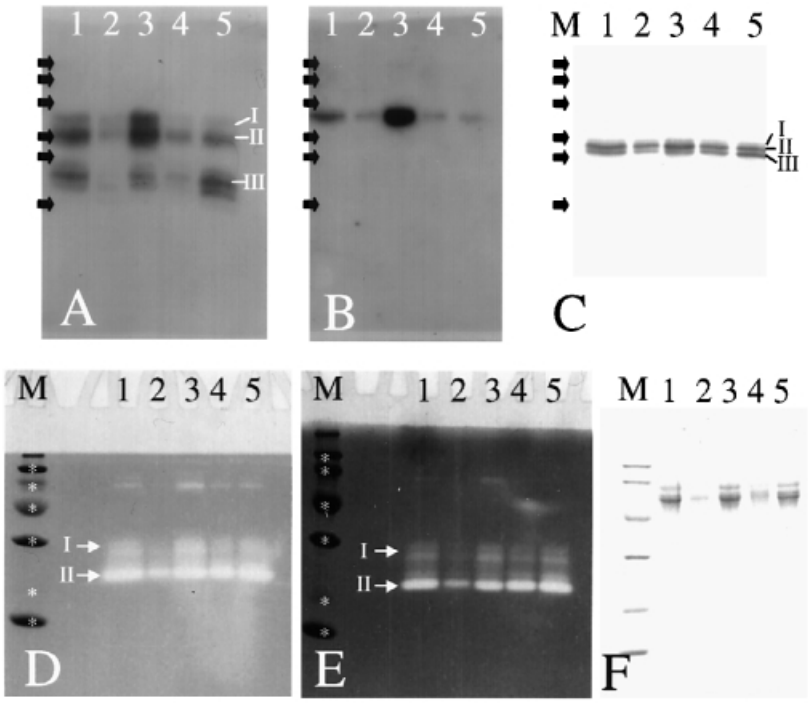

Figure 2. Analyses of different forms of SCTE and SCCE in superficial stratum corneum from individuals 1-5 by immunoblotting and zymography. Extracts of single tape strips were prepared as described under *Materials and Methods*. (*A–C*) Show the same immunoblot, prepared under reducing conditions, consecutively labeled with antibody Br-B1 (*A*), antibody Pe-C1 (*B*), and anti-SCCE (*C*). Secondary antibodies were in (*A*, *B*) peroxidase-conjugated and detected by ECL+, in (*C*) alkaline phosphatase-conjugated. Bound antibodies in one labeling were removed from the membrane before the next labeling. (*D*, *E*) Show zymograms developed at pH 8 and pH 5.5, respectively. (*F*) Coomassie stained SDS–PAGE gel with extracts in electrophoresis sample buffer with mercaptoethanol, prepared from the tape strips after the initial extraction with acetic acid. M in (*C–F*) molecular mass markers. *Arrows* in (*A–C*) molecular mass: from top 106, 80, 50, 33, 28, and 19 kDa, respectively. Asterisks in (*D*, *E*), and bands under "M" in (*F*): molecular mass: from top 93, 66, 45, 31, 22, and 14 kDa, respectively. Roman numbers in (*A*): I, pro-SCTE; II, SCTE; III, proteolytically modified SCTE. Roman numbers in (*C*): I, glycosylated pro-SCCE; II, unglycosylated pro-SCCE and glycosylated SCCE; III, unglycosylated SCCE. Roman numbers in (*D*, *E*): I, SCTE; II, SCCE.

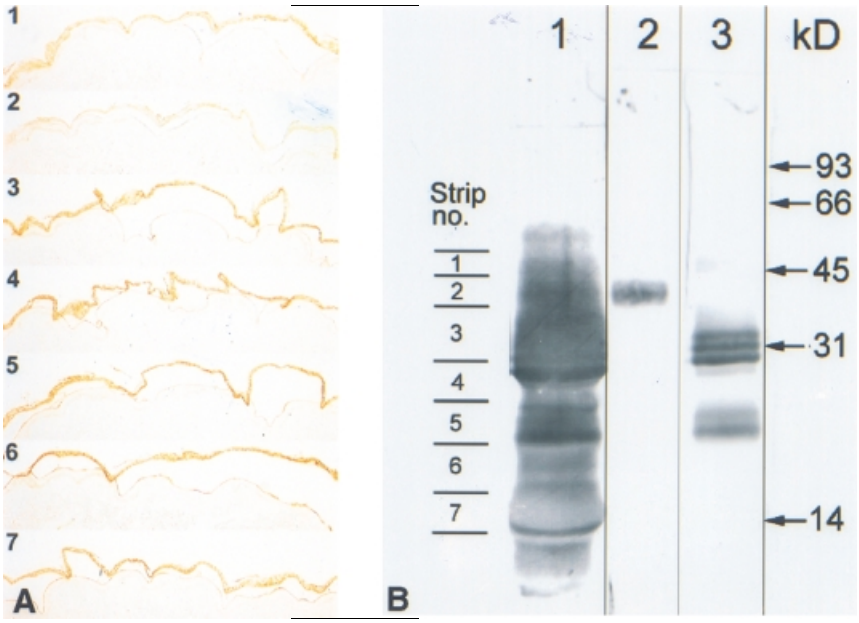

Figure 3. The antibody Pe-C1 is specific for pro-SCTE. (*A*) shows immunoperoxidase staining of normal human epidermis with Pe-C1 which had been adsorbed with horizontal strips nos 1–7, prepared as outlined in (*B*) (also see *Materials and Methods*). (*B*) shows strips of a PVDF membrane with proteins which had been electrophoretically transferred after SDS–PAGE under reducing conditions. *Lane 1*, Coomassie staining; partially purified, lyophilized and resolubilized material corresponding to 1.25 g of dried plantar stratum corneum was applied. *Lanes 2–3*, immunoblots with antibody Pe-C1 and anti-SCCE, respectively; secondary antibody alkaline-phosphatase-conjugated. Amount of material applied on each lane = 1/10 of amount applied on lane 1. Molecular mass markers to the right.

(**Fig 2*A–C***), zymography (**Fig 2*D–E***), and SDS–PAGE (**Fig 2*F***). Each immunoblot was consecutively labeled with antibody Br-B1 (suggested specificity for the catalytically active part of SCTE; **Fig 2*A***), antibody Pe-C1 (suggested specificity for the propeptide of SCTE; **Fig 2*B***), and antibodies specific for SCCE (**Fig 2*C***). All three antibodies detected components in all extracts. Br-B1 labeled components with molecular masses of about 33-37 kDa, and a group of components with molecular masses of about 25 kDa (**Fig 2*A***). Pe-C1 labeling was confined to components with a molecular mass of about 37 kDa (**Fig 2*B***). The SCCE-specific antibodies labeled a triplet of components with molecular masses of about 28-30 kDa (**Fig 2*C***).

In all five extracts proteolytic activity corresponding to SCCE (molecular mass of about 25 kDa; unreduced samples), and SCTE (molecular mass of about 30 kDa) could be detected on zymograms developed at pH 8 (**Fig 2*D***) as well as at pH 5.5 (**Fig 2*E***). The differences in intensity of proteolytic activity between individuals was reflected by the differences in amounts of keratins which could be extracted from the strips, as revealed by Coomassie-staining after SDS–PAGE of extracts prepared under reducing conditions in the presence of SDS (**Fig 2*F***).

Immunohistochemical analyses The antibodies Pe-C1 and Br-C10 (both with suggested specificity for pro-SCTE), gave positive staining of structures in normal human epidermis. No specific staining could be obtained with the antibody Br-B1.

In immunofluorescence microscopy of interfollicular epidermis Pe-C1 labeled a thin zone close to the transition between viable and cornified epidermis (**Fig 4*A***). With SCCE-specific antibodies

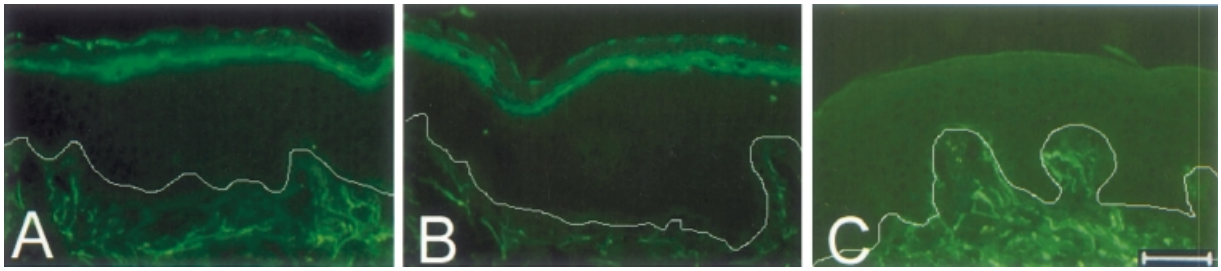

Figure 4. SCTE and SCCE in interfollicular epidermis. Immunofluorescence labeling. (*A*) Antibody Pe-C1 (6 μg per ml), (*B*) anti-SCCE (0.5 μg per ml), (*C*) control IgG (10 μg per ml). *Scale bar*: 50 μm.

the same part of the epidermis was stained (**Fig 4*B***). No specific labeling was seen with control IgG (**Fig 4*C***).

Immunoperoxidase staining of normal human epidermis with Pe-C1 is shown in **Figs 3**(***A***), **5**(***A***), **6**(***A***), **7**(***A***), and **8**(***A***). Staining with SCCE-specific antibodies is shown in **Figs 5**(***B***), **6**(***B***), **7**(***B***), and **8**(***B***). Staining with control IgG was always negative (not shown). Antibody Pe-C1 labeled a thin zone at the border between stratum granulosum and stratum corneum, but also the entire stratum corneum (**Figs 3*A*** and **5*A***). The SCCE-specific staining had a similar pattern, but the number of stained cell layers in the stratum granulosum was higher (**Fig 5*B***).

In order to study the specificity of Pe-C1, adsorption experiments were carried out. Whereas adsorption with recombinant pro-SCCE at low concentrations (0.06 μg per ml pro-SCCE; antibody concentration 0.5 μg per ml) effectively abolished the staining seen with the SCCE-specific antibodies, the staining with Pe-C1 was unaffected, also at the highest concentrations (60 μg per ml pro-SCCE; antibody concentration approximately 0.6 μg per ml) of pro-SCCE used (results not shown). An experiment in which PVDF strips with electrophoretically separated and transferred proteins from a reduced sample of a chromatography fraction containing SCTE as well as SCCE were used as adsorbents is shown in **Fig 3**. Only the strip containing material reacting with Pe-C1 on immunoblots (**Fig 3*B***) was able to abolish immunoperoxidase staining of normal epidermis and stratum corneum with Pe-C1 (**Fig 3*A***, strip 2). Strip 3, in spite of containing large amounts of material reacting with the SCCE-specific antibodies, was ineffective in adsorbing Pe-C1 antibodies. No strip prepared with reduced samples could abolish the labeling of the SCCE-specific antibodies (results not shown). With unreduced samples the strip containing SCCE protein was highly effective in this respect. The Pe-C1 labeling, on the other hand, could not be abolished with strips from unreduced samples. (Results from adsorption experiments with unreduced samples not shown.)

The comparison of the immunohistochemical labeling patterns of Pe-C1, suggested to be specific for pro-SCTE, and the SCCE-specific antibodies was extended to terminal hair follicles and sebaceous follicles from the scalp and the chest, in addition to interfollicular epidermis (see above). **Figure 7** shows results from the intermediate parts of a terminal hair follicle, and **Fig 8** from a sebaceous follicle from the scalp, and **Fig 6** from a terminal follicle at a level approximately halfway between the bulb and the opening of the sebaceous duct. In general, the staining patterns of the two antibodies were very similar. The staining obtained with Pe-C1 was usually weaker than with the SCCE-specific antibodies. The most obvious difference between the two antibodies was found in deeper parts of hair follicles. Whereas Pe-C1 staining was always confined to the inner root sheath (**Fig 6*A***), the SCCE-specific antibody labeled cells in the outer root sheath in addition to the inner root sheath (**Fig 6*B***). The results for the two antibodies are summarized in **Table III.**

DISCUSSION

There is reasonably good evidence in support of the hypothesis that SCCE may play an important part in desquamation (Egelrud *et al*, 1996). There is also, however, evidence that SCCE may not be the only protease involved in this process, and that other enzymes such as SCTE may be involved (Lundström and Egelrud, 1991; Egelrud, 1993; Suzuki *et al*, 1994, 1996). SCTE may itself take part in the degradation of intercellular cohesive structures in the stratum corneum. It is also a strong candidate for being responsible for the proteolytic activation of the SCCE-precursor. Until recently, knowledge about SCTE was limited to information obtained in enzymologic studies (Lundström and Egelrud, 1991; Egelrud, 1993; Suzuki *et al*, 1993, 1994, 1996). The recent successful purification and cDNA cloning of SCTE (Brattsand and Egelrud, 1999) have given new opportunities to characterize this enzyme and to elucidate its physiologic function.

In this study we have produced and characterized SCTE-specific antibodies, and used these antibodies in immunohistochemical and immunochemical analyses. *scte*-cDNA was produced from RNA from HaCaT cells and used to produce fusion proteins containing

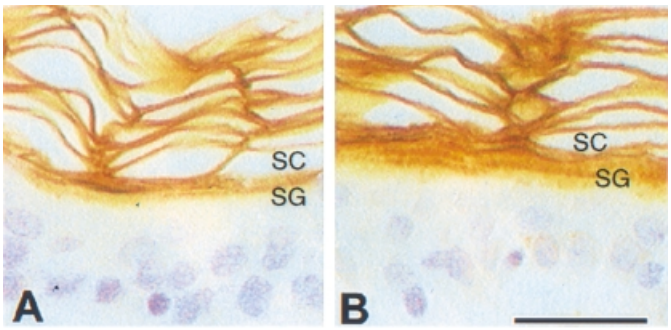

Figure 5. SCTE and SCCE in interfollicular epidermis. Immunoperoxidase staining. (*A*) Antibody Pe-C1 (6 μg per ml), (*B*) anti-SCCE (3 μg per ml). SC, stratum corneum; SG, stratum granulosum. *Scale bar*: 25 μm.

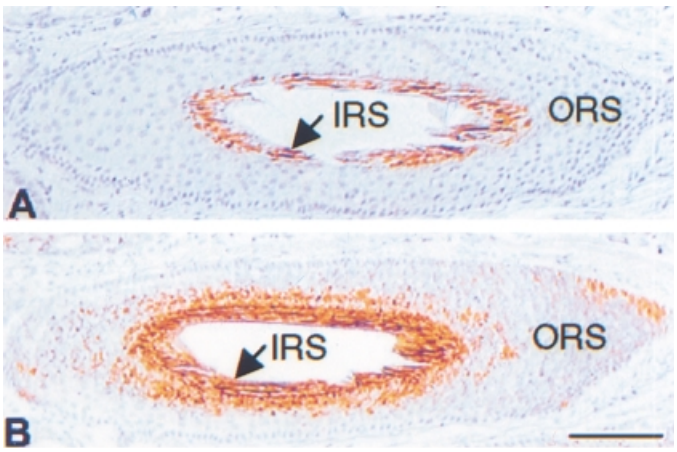

Figure 6. SCTE and SCCE in terminal hair follicle. Transversely cut serial sections of follicle from scalp, approximately halfway between bulb and opening of sebaceous duct. Immunostaining with antibody Pe-C1 (*A*) and anti-SCCE (*B*). Antibody concentrations as in **Fig 5**. IRS, inner root sheath; ORS, outer root sheath. *Scale bar*: 100 μm.

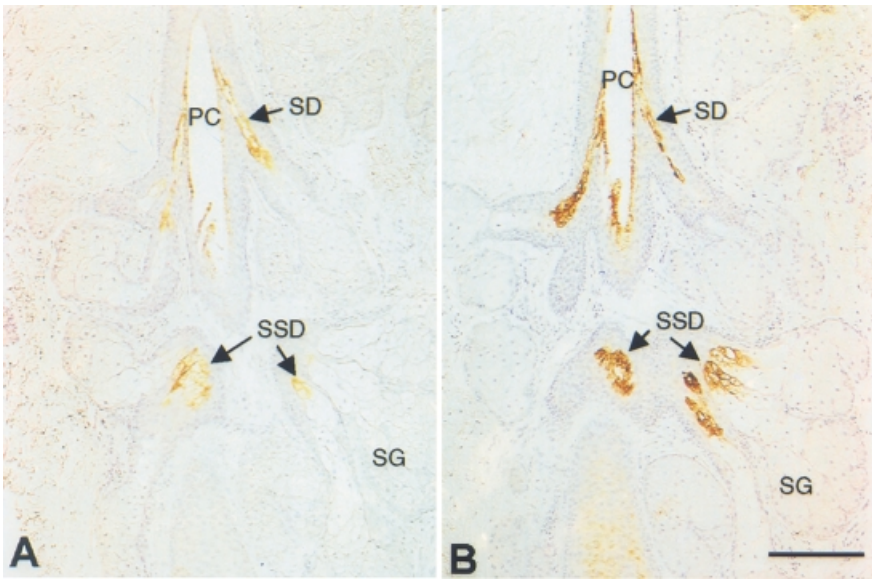

Figure 7. SCTE and SCCE in terminal hair follicle. Serial sections of intermediate parts of hair follicle from scalp. Immunostaining with antibody Pe-C1 (*A*) and anti-SCCE (*B*). Antibody concentrations as in **Fig 5**. PC, pilary canal; SD, sebaceous duct; SSD, secondary sebaceous duct; SG, sebaceous gland. *Scale bar*, 200 μm.

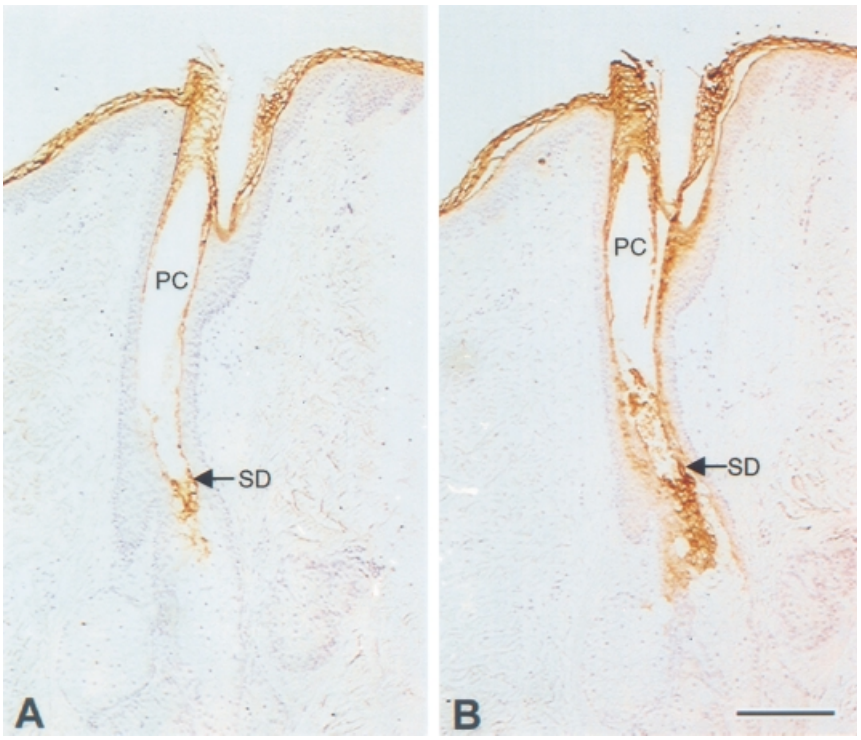

Figure 8. SCTE and SCCE in sebaceous follicle. Serial sections of sebaceous follicle from scalp. Immunostaining with antibody Pe-C1 (*A*) and anti-SCCE (*B*). Antibody concentrations as in **Fig 5**. PC, pilary canal; SD, sebaceous duct. *Scale bar*: 200 μm.

SCTE-derived polypeptides in bacteria, which were then used both for immunization and for affinity purification of antibodies. (At present we have no explanation to the fact that SCTE as well as SCCE are expressed by HaCaT cells, irrespective of state of differentiation, in spite of the fact that expression of these enzymes in the epidermis is seen only in high suprabasal cells.) In the ensuing characterization of the antibodies we paid special attention to questions concerning antibody specificity and possible cross-reactions.

As characterized by SDS–PAGE and amino acid sequence analyses, purified native SCTE has one N-terminal amino acid sequence, and a molecular mass of about 33 kDa in reduced form. In unreduced form its apparent molecular mass is approximately 30 kDa and it has two N-terminal amino acid sequences, one of which is the same as for reduced SCTE, and one which can be identified as an internal sequence of the cDNA-deduced amino acid sequence of SCTE (Brattsand and Egelrud, 1999). This suggests that the SCTE which can be purified from plantar stratum corneum is partially modified by proteolytic cleavage in its interior, and that the resulting polypeptides of the modified enzyme are held together by reducible cysteine bonds. The *scte*-cDNA predicts a 37 amino acid residues long propeptide of SCTE, suggesting that the enzyme is produced as an inactive precursor with a molecular mass of about 37 kDa.

Thus, on immunoblots of reduced samples prepared from a tissue containing SCTE and its precursor an antibody specific for the part of the SCTE molecule which corresponds to the catalytically active enzyme may be expected to label: (i) components with a molecular mass of about 37 kDa, corresponding to pro-SCTE; (ii) components with a molecular mass of about 33 kDa, corresponding to intact, active SCTE; and (iii) components with lower molecular mass, corresponding to proteolytically modified SCTE. The fact that this was exactly what was found in analyses of crude extracts of plantar stratum corneum with the polyclonal rabbit antibody Br-B1

Table III. Comparison of staining patterns of antibody Pe-C1 and SCCE-specific antibodies (immunoperoxidase)

Location	Pe-C1 (pro-SCTE)	SCCE
Interfollicular epidermis	One (?) cell layer in stratum granulosum; stratum corneum (**Figs 4*A*, 5*A***)	Several cell layers in stratum granulosum; stratum corneum (**Figs 4*B*, 5*B***)
Pilary canal	Narrow zone facing the lumen (**Figs 7*A*** and **8*A***)	Narrow zone facing the lumen (**Figs 7*B*** and **8*B***)
Common sebaceous duct	Narrow zone facing the lumen. Occasional staining of material within ducts (**Figs 7*A*** and **8*A***)	Narrow zone facing the lumen. Material within ducts (**Figs 7*B*** and **8*B***)
Secondary sebaceous ducts	Weak staining of luminal surface; occasional staining of material within ducts (**Fig 7*A***)	Luminal surface; material within ducts (**Fig 7*B***)
Inner root sheath	Stained (**Fig 6*A***)	Stained (**Fig 6*B***)
Outer root sheath	Not stained (**Fig 6*A***)	Cells close to inner root sheath stained (**Fig 6*B***)

gives strong evidence that this antibody is specifically reactive towards epitopes in the catalytically active part of SCTE. Likewise, the results with the antibodies Br-C10 and Pe-C1 are compatible with a specificity of these antibodies for epitopes specifically associated with pro-SCTE.

SCTE and SCCE are both serine proteases, and the amino acid sequences of their catalytically active parts show considerable homology (Hansson *et al*, 1994; Brattsand and Egelrud, 1999). It therefore seemed possible that antibodies raised against the two enzymes might cross-react. Immunoblot analyses, however, gave no evidence for cross-reactions. Neither could we find any evidence of cross-reactions in the adsorption experiments carried out in order to test the specificity of the antibodies when used in immunohistochemical analyses. Evidence in favor of a high specificity of the SCTE antibodies used in the immunohistochemical analyses also comes from the fact that the way they were prepared was designed in order to extract antibodies specific for the SCTE precursor, and that the amino acid sequence of the SCTE propeptide does not show significant homology to any other known proteins (Brattsand and Egelrud, 1999). We conclude that the antibodies prepared against SCTE show a high degree of specificity, and that there were no cross-reactions between these antibodies and the SCCE-specific antibodies.

A method was developed which made it possible to analyze stratum corneum obtained in one single tape strip of normal skin by zymography as well as several consecutive immunoblot analyses. In samples from all individuals analyzed active SCCE and SCTE could be detected at pH 8 as well as at pH 5.5. Thus, SCTE may be active at the physiologic pH of the stratum corneum (Öhman and Vahlquist, 1994). In immunoblot analyses all samples were shown to contain components corresponding to active SCTE as well as pro-SCTE, in addition to active SCCE and SCCE precursor. This implies that the staining of the entire stratum corneum with the antibody Pe-C1 was not due to the labeling of free SCTE propeptide, but that it was at least partially due to the labeling of intact pro-SCTE molecules.

With few exceptions the expression patterns of SCTE and SCTE in normal epidermis were identical. This means that SCTE, as has previously been shown for SCCE, may be exclusively expressed at sites where epidermal cells are cornified and eventually shed, i.e., at sites where there is a need for a desquamation-like process to occur.

We obtained evidence that even the most superficial parts of interfollicular stratum corneum contains significant amounts of the catalytically inactive precursors of SCTE and SCCE, in addition to the active forms of the two enzymes. We also showed that SCTE as well as SCCE are active not only at weakly alkaline pH, but also at pH values as low as 5.5.

Our results thus suggest two possible mechanisms by which the rate of desquamation may be regulated, i.e., by the extent of activation of protease precursors, and by changes in pH of the stratum corneum extracellular space. An obvious possibility is that SCTE and SCCE may be parts of a protease cascade of which several members still remain to be discovered.

In conclusion, this study has presented some properties of what may be one of the missing links in our understanding of the desquamation process.

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